International Journal of Medicine and Pharmaceutical Sciences (IJMPS)
ISSN(P): 2250-0049; ISSN(E): 2321-0095
Vol. 4, Issue 1, Feb 2014, 49-58

CHEMOPREVENTIVE POTENTIAL OF DIOSGENIN AGAINST 7, 12-**DIMETHYLBENZ (A) ANTHRACENE (DMBA) INDUCED SKIN CARCINOGENESIS IN MICE

MANOJ KUMAR DAS & RUPJYOTI BHARALI

Department of Biotechnology, Gauhati University, Guwahati, Assam, India

ABSTRACT

Cancer chemoprevention by natural and synthetic agents offers significant promise for reducing the incidence and mortality of cancer.In this regard, diosgenin, [(25R)-Spirost-5-en-3-beta-ol, 22alpha-Spirost-5-en-3beta-ol] was tested against 7, 12-dimethylbenz[*a*]anthracene (DMBA)-croton oil model of skin carcinogenesis in Swiss albino mice. The topical application of 5mg/Kg body weight diosgenin showed significant reduction in tumor yield, tumor burden and cumulative number of papillomas in Group II, III and IV mice in comparison to the carcinogen control. Elevation in the percent inhibition of tumor multiplicity and latency in tumor appearance were evident. Histological studies depicted change in both the epidermis and the dermis of treated animals. All the diosgenin treated groups documented regression of neoplastic changes. Scanning electron micrograph revealed regions of comparatively reduced keratinization and sub-normal epithelial surface compared to damaged surface in carcinogen control. Transmission electron micrograph of carcinogen control mice recorded disrupted epithelial cells, ruptured cell membrane and rigorous impact on mitochondria that were reduced significantly when treated with diosgenin. This controlled study on animal model identifies the chemopreventive efficacy of diosgenin for appropriate testing of hypothesis based on observational studies on humans.

KEYWORDS: Diosgenin, Cancer Chemoprevention, DMBA, Skin Carcinogenesis

INTRODUCTION

In recent years, growing interest has been focused on the field of cancer prevention. Cancer chemoprevention is defined as the use of chemical agents in healthy individuals to block, reverse, or delay the development of invasive cancer. Cancer prevention by chemopreventive agents offers significant promise for reducing the incidence and mortality of cancer.

Carcinogenesis in general is a complex multi-step process that requires the accumulation of multiple genetic and epigenetic events (Hahn and Weinberg, 2002). In multi step carcinogenesis process, the inhibition of tumor promotion is regarded as an effective strategy for cancer chemoprevention, because tumor promotion occurs by repetitive exposure to tumor promoters in long term (Murakami et al., 1999).

Skin cancer chemoprevention is regarded as a useful model for cancer chemoprevention in general (Richmond and Viner, 2003). Amongst many poly aromatic hydrocarbons that are used in the etiology of skin cancer, 7, 12-dimethylbenz[*a*]anthracene (DMBA) is the most potent initiator that has ability to damage DNA and produce cancer in lesser time than any others (Slaga, 1984). Croton oil is the most potent promoting agent for the mouse skin hyperplasia. The active component of croton oil is 12-O-tetradecanoylphorbol-13-acetate (TPA), is a diester of phorbol and a potent tumor promoter often employed in biomedical research to activate the signal transduction enzyme protein kinase C (PKC) (Neidel et al., 1983).

The 7, 12-dimethylbenz[*a*]anthracene (DMBA)- 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) model of skin carcinogenesis involves initiation, promotion and progression and provides ample scope for studying the stage specific effect of chemopreventive compound (Slaga, 1984). Chemical carcinogenesis in mouse skin continues to help in the identification of important molecular and immunological pathways involved in cutaneous malignancy (Filler et al., 2007).

Diosgenin, also known as (25R)-Spirost-5-en-3-beta-ol, 22alpha-Spirost-5-en-3beta-ol and nitogenin, a steroidal sapogenin is a product of hydrolysis of saponin. Diosgenin has been shown to possess wide range of activities from various sources. It naturally occurs in *Costus speciosus, Smilax menispermoidea,* species of *Paris, Trigonella, Trillium* and many species of *Dioscorea.* Since first isolated from *Dioscorea tokoro* in the 1930s (Yang, 1981), diosgenin became very important compound in the pharmaceutical industry as it is a natural source of various steroidal drugs. It can be absorbed through gut and plays an important role in the control of cholesterol metabolism (Roman et al., 1995). Estrogenic effects of diosgenin have also been demonstrated by several workers (Derrida, 2003; Rao et al., 1992). Diosgenin has shown regenerative effects on skin aging (Tada et al., 2009). Diosgenin has shown antiviral potential *in vitro* against hepatitis C virus.It is also used as a dietary supplement, in combination with interferon-α, exerted an additive effect on the resultant anti-HCV activity (Wang et al., 2011). Recently, anti-thrombosis effect of diosgenin extract from *Dioscorea zingiberensis* is reported (Gong et al., 2011).

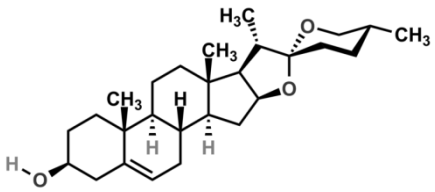

Figure 1: Chemical Structure of Diosgenin [(3β, 25*R*)-spirost-5-en-3-ol]

All these observations warrant a possible link between diosgenin and prevention of skin carcinogenesis. Therefore, the present study was an endeavor to examine the chemopreventive potential, if any of diosgenin using two stage skin carcinogenesis model in Swiss albino mice.

MATERIALS AND METHODS

Test Material

Diosgenin [IUPAC name: (3β, 25R)-spirost-5-en-3-ol; $C_{27}H_{42}O_3$, (414.62 g mol^{-1}), E.C. 208-134-3] was purchased from Sigma-Aldrich Co., St. Louise, USA (lot no.079K1096). Diosgenin was applied topically at a dose of 5mg/Kg body weight of mice.

Test Animals

Randomly bred Swiss albino mice were *(Mus musclus)* maintained in the animal house, Department of Biotechnology, Gauhati University, Guwahati and sacrificed as per the guidelines of Animal Ethical Committee of Gauhati University (Regd. No.902/AC/05/CPCSEA). Both male and female mice of 5-6 weeks of age weighing about 20± 2g were used. The hairs on the dorsal side of the mice were clipped off before the commencement of the experiment and kept under observation for 7-10 days. Mice that showed no or minimum hair reappeared were selected for the study.

Experimental Design- DMBA Induced Skin Carcinogenesis

Mice were segregated in four groups of 6 animals each. All groups received topical application of initiator, DMBA and promoter, croton oil. The effect of vehicle was observed for 7 consecutive weeks without any apparent change in dorsal skin of mice, hence not continued further (Bharali et al., 2003). Details of treatment are presented in Table 1.

The chemopreventive potential of diosgenin was investigated by the method of Berenblum and Shubik (1947) for 15 consecutive weeks.

The parameters that were studied includes cumulative number of papillomas, tumor incidence (percentage of papilloma bearing mice), tumor yield (average number of papillomas per mouse), tumor burden (papillomas per papilloma bearing mouse), percent inhibition of tumor multiplicity and average latent period of tumor occurrence.

Table 1: Experimental Design- DMBA Induced Skin Carcinogenesis in Swiss Albino Mice

Group	No. of Animal	Treatment
I (Control)	6	Single topical application of DMBA (0.1%), followed by croton oil (1% in acetone) application thrice a week till the end of the experiment.
II	6	DMBA and croton oil as in group I + topical application of diosgenin (5mg/Kg b.w. in acetone/day) for 14 days (7 days before & 7 days after DMBA application).
III	6	Topical application of diosgenin (5mg/Kg b.w. in acetone/day) for 91 days (from croton oil treatment). DMBA and croton oil as in group I.
IV	6	Topical application of diosgenin (5mg/Kg b.w. in acetone/day) for 105 days (7 days before DMBA application and continued to end of experiment). DMBA and croton oil as in group I.

Statistical Analysis

All the results are expressed as mean $\pm$ S.D. of 6 animals. Statistical differences between the experimental groups were determined by One Way Analysis of Variance (ANOVA) followed by Dunnett test at 0.05 significance level using Graph Pad Prism ver. 5.03, Graph Pad Software, San Diego, California, USA.

Histological Study

To further investigate the effect of diosgenin on 7, 12-dimethylbenz[a]anthracene (DMBA) induced skin carcinogenesis, the animals were sacrificed at the end of the experiment (after 15 weeks) and dorsal skin of mice was considered for histological study. Affected skin, skin with papillomas as well as skin of normal mice were placed in formalin and dehydrated with graded alcohols. Tissues were embedded in paraffin after xylene treatment. Serial microtome sections were stained in haematoxylin and eosin to differentiate the nucleus and cytoplasm respectively (Kehar and Wahi, 1967).

Scanning Electron Microscopic Study

To investigate any kind of change in surface organization during experimental induction of skin carcinogenesis, dorsal skin of mice was considered for scanning electron microscopic study (SEM Model: JSM-6360 –JEOL). Tissues were primarily fixed in Karnovsky's Fixative (Karnovsky, 1965). Dehydration was carried out in graded doses of acetone and dried in Tetra Methyl Sialane (Dey et al., 1989). The specimens were mounted on brass stubs and silver coated.

Transmission Electron Microscopic Study

To investigate any kind of change in internal organization of the cells during experimental induction of skin carcinogenesis, dorsal skin of mice was considered for transmission electron microscopic study (TEM Model: JEM-2100). Tissues were fixed in Karnovsky's Fixative (Karnovsky, 1965). Dehydration was carried out in graded doses of acetone and the samples were transferred into BEEM® capsules and polymerized. Ultrathin sections obtained with RMC MTX cryo- ultramicrotome were collected onto grids. Grids were double stained using uranyl acetate and Reynold’s lead citrate.

RESULTS AND DISCUSSIONS

The chemopreventive effect of diosgenin on DMBA induced skin carcinogenesis was determined in terms of papillomas appearing on the shaven back of Swiss albino mice. Papillomas that persisted for two weeks or more were recorded at weekly intervals. Results of parameters studied have been recorded in the Table 2. The topical application of diosgenin did not affect the body weight during the experimental period. Papilloma started appearing from 6-12 weeks during exposure to the initiator and promoter depending on treatment groups.

Table 2: Chemopreventive Potential of Diosgenin on DMBA Induced Skin Papillomagenesis in Mice

Group	Body Weight (g) (Mean ± S.D.)		Cumulative Number of Papillomas	Tumor Incidence (%)	Tumor Yield	Tumor Burden	% Inhibition of Tumor Multiplicity	Average Latent Period
	Initial	Final						
I (N=6)	19±1.83	21.1±2.07	107	100	10.7	10.7	---	5.4
II (N=6)	18±1.61	22.3±1.30	81	79.7	8.1*	8.1*	53	6.6
III (N=6)	19±1.12	22.6±2.05	37	54	3.7*	4.5*	76	9.6
IV (N=6)	19±1.68	23.2±1.45	22	41.4*	2.2*	2.9*	85	11*

N in parenthesis=Number of mice in each group; *significancant among different groups at P<0.05

The application of diosgenin showed significant reduction in tumor yield, tumor burden and cumulative number of papillomas in Group II, III and IV in comparison to the carcinogen control (Group I). Elevation in the percent inhibition of tumor multiplicity and marked latency in the appearance of tumors were noticed in all the modulator treated groups. Similar chemopreventive potential of citrus pulp and juices (Tanaka et al., 2012a), carotenoids (Tanaka et al., 2012b), synergy1 and soybean (Gourineni et al., 2011), a plant coumarin from *Gelsemium sempervirens* (Bhattacharyya et al., 2010) against 7,12-dimethylbenz[a]anthracene induced skin carcinogenesis are in recent literature. Fenugreek seeds that contain the steroidal sapogenin, diosgenin have shown similar protective effect against 7, 12-dimethylbenz[a] anthracene (DMBA) in rats (Amin et al., 2005).

In a study carried out by McLean et al. (2001), fak+ / - heterozygous mice displayed reduction in the number of papilloma when treated with 7, 12 dimethylbenz[a]anthracene and was correlated with reduced Focal Adhesion Kinase (FAK) protein expression in the skin. 7, 12-dimethylbenz[a]anthracene induces a mutation of H-ras and provides possible explanation for suppression of papilloma formation when FAK protein is limiting.

Histological Findings

At the end of the experiment, sections of skin from interscapular papilloma of mice and skin of normal mice were processed for histopathological studies. In the untreated mice, normal cellular architecture of the skin was observed (Figure 2A).

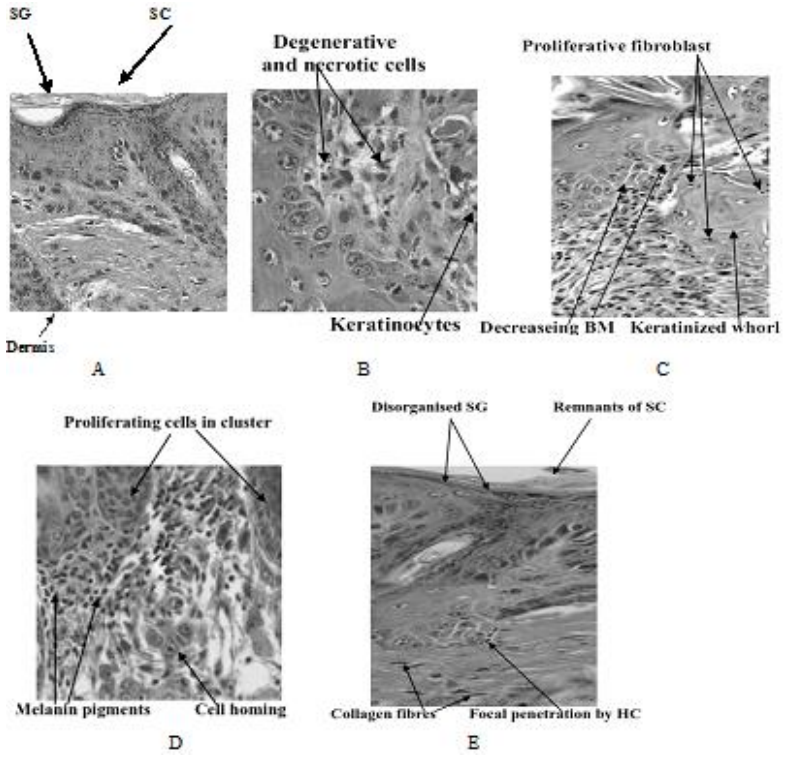

Figure 2: Histopathology of Dorsal Skin of Swiss Albino Mice. A- Normal (Negative Control), B- Group I (Positive Control), C-Group II, D - Group -III, E-Group IV. Original Magnification: 100 ×

Group I (control) animal skin showed presence of keratinized tissue along with hyper-plastic stratified squamous epithelial cells, increase in dermal inflammatory infiltrate, proliferation of basal epidermal layer. Central mass of degenerative and necrotic cells surrounded by neoplastic cells were also evident (Figure 2B). The keratins of the papillomas display greater heterogeneity, particularly among the high-molecular-weight keratins (Nelson and Slaga, 1982). In Group II animals, the dermo-epidermal junction showed gross disorganization of surrounding connective tissue, basement membrane (BM) material was decreasing, proliferated fibroblasts were noticed along with keratinized whorl (Figure 2C). Similarly, Tarin (1967) has observed small gaps in the basement membrane accompanied by the accumulation of fragmented material in mice skin papillomagenesis. Group III animals depicted pleomorphism of the proliferating cells arranged in clusters and excessive melanin pigments and cell homing (Figure 2D). In Group IV animals, remnants of *stratum corneum* (SC) could only be seen with disorganised s*tratum granulosum* (SG) layer. The *stratum basale* depicted regular collagen fibres in the dermis layer and focal penetration by some hyper plastic cells (HC) (Figure 2E). Devasena et al. (2003) reopted that decreasing the expression of phospholipase A and C are responsible for prevention of tumor formation and improving the histological features including dysplasia and cellular pleomorphism.

Scanning Electron Microscopic Findings

Dorsal skin of normal mice showed distinct well aligned surface with intact hair in and cornified layer (Figure 3A).

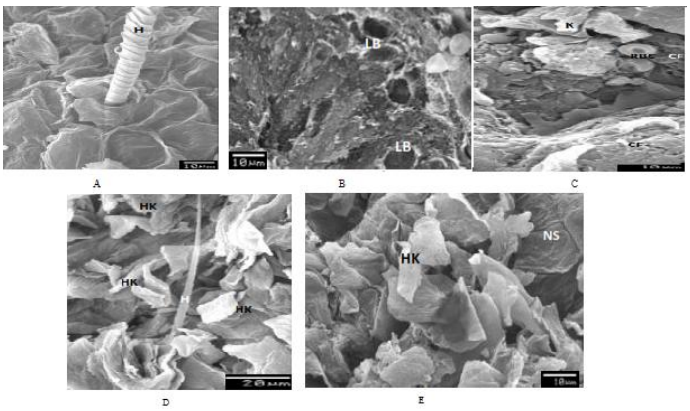

Figure 3: Scanning Electron Micrograph of Mouse Dorsal Skin: A-Normal, B-Group I, C-Group II, D- Group III, E-Group IV. LB-Lipocytes Boundary, H-Hair, K-Keratinization, RBC-Red Blood Cell, CF- Collagen Fibres, HK-Hyper-Keratosis, NS- Normal Surface

Group I (carcinogen control) animal micrograph revealed no integrity among surface epithelial cells. A no. of lesion with damaged lipocytes boundaries (LB) could be seen (Figure 3B). Chauhan et al. (1993) observed necrotic epidermal cells with breakage in delicate collagenous structure surrounding the lipocytes on mouse skin.

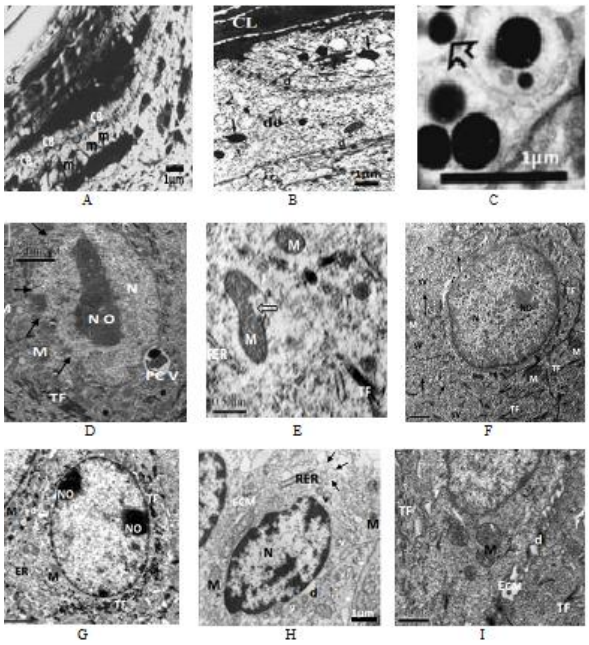

Figure 4: Trasmission Electron Micrograph of Mouse Dorsal Skin: A-Normal, B, C-Group I, D, E-Group II, F, G-Group III, H, I-Group IV. CL- Cornified Layer, CB-Cell Boundary, D-Desmosomes, M-Mitochondria, De-Dermis, Double Arrow-Inclusion Bodies, N- Nucleus, NO- Nucleolus with Degeneration of Nuclear Membrane (Arrow), PCV-Phagocytic Vacuole, Open Arrow- Degenerated Mitochondria, TF-Tonofilaments, RER-Rough Endoplasmic Reticulum, SV- Small Vesicle, ER- Endoplasmic Reticulum, ECM-Extra Cellular Matrix, V- Vesicle, Close Headed Arrow- Free Ribosomes

Group II animals showed disrupted collagen fibers, reduced keratinization and damaged surface with red blood cells (Figure 3C).Tarin (1967) observed a decrease in the size of collagen fibres during skin tumor promotion. Group III animals depicted regions of hyper- keratinization (Figure 3D). However, Group IV animals also showed hyper-keratinization but some regions revealed completely normal epithelial surface (Figure 3E).

Transmission Electron Microscopic Findings

Figure 4(A-I) are transmission electron micrograph of experimental animals. Normal mice depicted regular structure of epidermis and dermis. Carcinogen control mice showed disrupted epithelial cells with focal thickening and

ruptured cell membrane. Swollen mitochondria with multiple round electron-dense inclusion bodies and loss of cristae were spotted. Rajeswari et al. (2003) observed papillomatous skin tumor samples with similar focal parakeratosis and acanthosis of the epidermis. Group II mice revealed increased intercellular gap, degeneration of nucleus, disintegrating nucleolus, lysosome forming phagocytic vacuole. Group III animals recoded increased number of membrane bound small vesicle, intact endoplasmic reticulum, mitochondria and smaller size nucleolus. The decrease in nucleolus size was also observed in a study which was correlated to positive effect of the modulator (Raick and Burdz, 1973). Group IV animals had nucleated and anucleated layers similar to normal skin with tight adherence between keratinocytes of different layers. Large number of cells showed distinct cell organelles like nucleus, mitochondria with cristae, desmosomes, free ribosomes and extra cellular matrix which suggests protective role of diosgenin from carcinogenic effect of DMBA. Raick and Burdz (1973) had also proposed that appearance of compact cytoplasm with intact mitochondria may protect the cell from the carcinogenic effect of DMBA.

CONCLUSIONS

Cancer chemoprevention is a pharmacological approach to intervene with the objective of arresting or reversing the process of carcinogenesis. The reversibility of tumor promotion provides an opportunity to interrupt or delay the development of altered lesions resulting in tumor formation. The chemoprevention seeks to eliminate precancerous cells in order to avoid the necessity of chemotherapy. Therefore, assessment of chemopreventive potential of diosgenin bears a significant value. A dose of 5mg/Kg body weight/day for 15 weeks at peri-initiational stage, at promotional stage and together at peri and post initiational stages revealed a significant reduction in tumor yield, tumor burden and cumulative number of papillomas in all the diosgenin treated groups. An elevation in the percent inhibition of tumor multiplicity and latent period of tumor occurrence were also recorded. Histological and ultra-structure analysis further confirmed that diosgenin is highly effective against DMBA induced skin carcinogenesis.

Thus, new compounds with cancer chemoprevention potential can be assessed on animal carcinogenesis model. In near future, chemopreventive strategies must identify subsets of the population at greater risk for cancer development and should target toward those groups. However, interpolation of outcome from animal studies to humans requires caution.

REFERENCES

1. Amin, A., Alkaabi, A., Al-Falasi, S. and Daoud, S.A. (2005). Chemopreventive activities of Trigonella foenum graecum (Fenugreek) against breast cancer. Cell Biol. Int., 29,687-94
2. Bernam, D., Hu, Y., Joubeh, S., Choi, Y.W. ,Menezes, D.W., O'Brien, T., Uitto, J., Rodeck, U. and Mahoney, M.G. (2007). Suprabasal Dsg2 expression in transgenic mouse skin confers a hyperproliferative and apoptosis-resistant phenotype to keratinocytes. J. Cell Sci., 120,758-771
3. Berenblum,I.and Shubik, P. (1947). A new quantitative approach to study the stages of chemical carcinogenesis in the mouse's skin. British J. Cancer.1, 383
4. Bharali, R., Tabassum, J. and Azad, M.R.H.(2003). Chemopreventive action of Phyllanthus urinaria Linn on DMBA induced carcinogenesis in mice. Indian J. of Experimental Biology, 41, 1325-1328
5. Bhattacharyya, S.S., Paul, S., Dutta, S., Boujedaini, N., Khuda-Bukhsh,A.R. (2010). Anti-oncogenic potentials of a plant coumarin (7-hydroxy-6-methoxy coumarin) against 7, 12-dimethylbenz [a] anthracene-induced skin papilloma in mice: the possible role of several key signal proteins. Zhong Xi Yi Jie He Xue Bao, 8(7),645-54

6. Chauhan, R.S., Murthy, L. V. R. and Malhotra, R. C. (1993). Effect of Sulphur Mustard on Mouse Skin--an Electron Microscopic Evaluation. Environ. Contam. Toxicol., 51,374—380

7. Derrida, M. (2003). Usages of Diosgenin from Wild Yam. (www.mdidea.com/products/ monomer/mono02.html). Accessed on 04.04.2012

8. Devasena,T., Gunasekaran,G., Viswanathan, P., Menon, V.P. (2003). Chemoprevention of 1, 2-dimethyl hydrazine-induced colon carcinogenesis by seeds of Trigonella foenum graceum L, Biologia. Bratislava., 58, 357-364

9. Dey, S., Basu, T.S., Baul, Roy, B.,Dey, D. (1989). A new rapid method of air drying for Scanning Electron Microscopy using Tetra Methyl Sialane – Short technical note. J.Microscopy,156 (2),259-261

10. Filler, R.B.,Roberts, S.J. and Girardi, M.(2007).Cutaneous Two-Stage Chemical Carcinogenesis. Cold Spring Harb. Protoc., doi:10.1101/pdb.prot4837

11. Gong, G., Qin, Y. and Huang, W. (2011). Anti-thrombosis effect of diosgenin extract from Dioscorea zingiberensis C.H. Wright in vitro and in vivo. Phytomedicine, 18(6), 458–463

12. Gourineni, V. P., Verghese, M., Boateng, J., Shackelford, L. and Bhat, K. N. (2011). Chemopreventive Potential of Synergyl and Soybean in Reducing Azoxymethane-Induced Aberrant Crypt Foci in Fisher 344 Male Rats. Journal of Nutrition and Metabolism. 2011, Article ID 983038, 8 pages http://dx.doi.org/10.1155/2011/983038

13. Hahn, W. C. and Weinberg, R. A. (2002). Modeling the molecular circuitry of cancer. Nat.Rev. Cancer 2, 331-341

14. Karnovsky, M. J. (1965). A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy.J. Cell Biol., 27,137A

15. Kehar, U. and Wahi, P.N. (1967). Cytologic and histologic behavior patterns of the premalignant lesions of the cervix in experimentally induced cervical dysplasia. Acta Cytologica, 11,1-15

16. Murakami, A., Ohigashi, H., Koshimizu, K. (1999). Chemoprevention: Insights into biological mechanisms and promising food factors. Food Rev. Int., 15, 335-395

17. Nelson, K.G. and Slaga, T.J. (1982). Keratin Modifications in Epidermis, Papillomas, and Carcinomas during Two-Stage Carcinogenesis in the SENCAR Mouse. Cancer Res., 42, 4176-4181

18. Niedel, J.E., Kuhn, L.J., Vandenberg, G.R. (1983). "Phorbol Diester Receptor Copurifies with Protein Kinase C". Proceedings of the National Academy of Sciences, 80, 36–40

19. Raick, A.N. and Burdz, K. (1973).Ultrastructural and biochemical changes induced in mouse epidermis by a hyperplastic agent, ethylphenylpropionate.Cancer Res., 33,2221-2230

20. Rajeswari, M.R., Jain, A., Sharma, A., Singh, D., Jagannathan, N. R.,Sharma, U. and Degaonkar, M. N. (2003). Evaluation of Skin Tumors by Magnetic Resonance Imaging. Laboratory Inbestigation, 83(9), 1279-1283

21. Rao, A.R. and Kale, R.K. (1992).Diosgenin-a growth stimulator of mammary gland of ovariectomized mouse. Indian J. Exp. Biol., 30,367-370

22. Richmond, E. and Viner, J.L. (2003). Chemoprevention of skin cancer. Semin Oncol Nurs.,19,62-69

23. Roman, I.D., Thewles, A., Coleman, R. (1995). Fractionation of livers following diosgenin treatment to elevate biliary cholesterol. Biochim. Biophys. Acta, 1255,77

24. Slaga, T.J., (1984).Multistage skin carcinogenesis: a useful model for the study of the chemoprevention of cancer. Acta Pharmaco Toxicol (Copeh), 55suppl (2),107-124

25. Tada, Y., Kanda, N., Haratake, A., Tobiishi, M., Uchiwa, H. and Watanabe, S. (2009). Novel effects of diosgenin on skin aging. Steroids, 74(6), 504-511

26. Tanaka, T., Tanaka, T., Tanaka, M. and Kuno, T. (2012a). Cancer Chemoprevention by Citrus Pulp and Juices Containing High Amounts of ß-Cry ptoxanthin and Hesperidin. Hindawi Publishing Corporation, Journal of Biomedicine and Biotechnology, 2012, 10 pages, Article ID 516981, http://dx.doi.org/10.1155/2012/516981

27. Tanaka, T., Shnimizu, M., Moriwaki, H. (2012b). Cancer Chemoprevention by Carotenoids. Molecules, 17, 3202-3242

28. Tarin, D. (1967). Sequential electron microscopical study of experimental mouse skin carcinogenesis. Int. J. Cancer, 2, 195–211

29. Wang, Y.J., Pan, K.L., Hsieh, T.C., Chang, T.Y., Lin, W.H., Hsu, J.T. (2011). Diosgenin, a plant-derived sapogenin, exhibits antiviral activity *in vitro* against hepatitis C virus. J. Nat. Prod., 74(4), 580-4. Epub 2011 Mar 10.

30. Yang, M.H. (1981). Steroidal sapogenins from plants of Dioscorea. Chin.Trad. Herb Drugs, 12, 41-48